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The cohesin subunit RAD21L functions in meiotic synapsis and exhibits sexual dimorphism in fertility

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Review timeline:

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been evaluated by four referees with respective expertise in the cohesin and meiosis fields, and I am pleased to inform you that all of them consider your knock-out study on the recently discovered meiotic RAD21L kleisin both important and interesting. We shall therefore be happy to consider the manuscript further for publication, pending adequate revision.

As you will see from the reports below, the major issues raised by all referees are not of technical nature but pertain to the writing and presentation of the study. I should nevertheless stress that this is not a trivial point in this case, as all referees shared these concerns and wrote down detailed comments on the issue. In this situation, it may appear warranted to completely re-write the paper before resubmission, in the spirit of the referees' comments. I prefer not to go into further detail at this point in my letter, given that especially the first three referees offer very explicit and constructive suggestions in this respect. However, I would strongly recommend that you have the final version carefully proofread and edited by colleagues both for language issues (ideally by a native speaker) and for scientific accessibility and readability (maybe involving also someone outside the immediate field?).

With regard to experimental revisions, the referees only ask for a few minor additions, which I agree will in most cases help to round up the study. There are two points that deserve further attention:
- the IF data intended on clarifying the lingering discrepancies about RAD21L localization: although the referees doubt that IF or similar techniques can clarify these issues in a fully decisive manner, especially referees 1 and 2 offer some suggestions for elucidating the basis of the controversial observations somewhat further - e.g. by testing lower antibody concentrations, and by providing

better characterization of the RAD21L antibodies employed. Such data should be included in the revision.
- referee 3 suggests a ChIP-dot blot analysis of the observed telomere attachment phenotype, which I feel may go beyond the scope of the current paper. Here, I would find it acceptable if you decided to leave this for a future, more focussed study on the telomeric roles, and in turn de-emphasized this part in the current paper as suggested by the reports.

We generally allow three months as standard revision time, but given the timeliness of the current study, I am hoping you will be able to resubmit a revised manuscript already considerably earlier. In any case, it is our policy that competing manuscripts published during the revision period will have no negative impact on our final assessment of your revised study (however, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed). Finally, I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Please also bear in mind that your letter of response will form part of the Review Process File, and will therefore be available online to the community (for more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html).

Should you have any additional question regarding this decision or your revision, please do not hesitate to contact me. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Recently, three papers have appeared reporting RAD21L, a novel meiosis-specific kleisin subunit of cohesin complexes, together with its cytological localization. The present manuscript provides the hoped-for complementation by describing the knockout phenotypes of rad21L. Given the importance of cohesins and the fact that only by removing or inhibiting a protein can we come to know its function, this paper makes an important contribution.

The paper consists of two major parts. One is a relatively straight-forward report of knockout phenotypes which clearly confirm the proposed role of RAD21L (or RAD21L-containing cohesin complexes) in DSB repair, homology searching and synapsis. A remarkable find is that rad21l-/- meiosis is normal in young female mice, but that lack of the protein confers an age-dependent affect on fertility.
The other part is basically a repeat of studies on the cytological localization of RAD21L. This part is justified since two previous papers by two other groups presented partially contradicting results and here, confirmation is provided for the observations in one of the two. However, in one crucial detail, the present manuscript differs from both previous reports. They had described an alternating localization of RAD21L and REC8 along meiotic chromosome axes, whereas here, they are shown to form continuous overlapping stretches. On the basis of the alternating pattern of RAD21L and REC8, one group put forward a model of a locus specific barcode of distribution of the two kleisins that would be shared between homologous chromosomes and provide a means of homology alignment or recognition. Since this is a strong statement, a more rigorous effort should be made here to provide explanations for the controversial observations.
I was wondering if the authors could try to apply antibodies at a lower concentration, since it is possible that immunostaining intensity depends on slightly varying densities of proteins along axial/lateral elements. These differences might be revealed only if antibody binding is not oversaturated.

While the data presented and the conclusions drawn here are valid and scientifically sound, the manuscript is poorly written. The writing style is overly wordy and convoluted, thus obfuscating rather than clarifying the work. The manuscript would greatly benefit from extensive editing by a native English speaker to present the work clearly and directly.
Also, readability would be improved by a more structured presentation of results and discussion. Subtitles should be used in the corresponding Results & Discussion section. Such chapters could follow the current organization of the section and include: Immunolocalization of the RAD21L protein. Gene disruption of rad21l. SC morphology and synapsis in mutant spermatocytes. Cohesion complexes in mutant spermatocytes. Telomere behaviour in mutant spermatocytes. Defective DSB processing in the mutant. Meiosis in mutant females.
Finally, in many parts, the manuscript is too copious, e.g., with the description of the testicular histology of rad21L-/- mutant mice, when it is anyway made clear later that the observed anomalies are due to a failure in pairing, SC formation and DSB repair (which are known to lead to apoptosis and azoospermia). Parts of the manuscript read like a review article as much of the discussion barely relates to the findings reported here.

Specific comments:
p. 4, line 20, the yeast mutant should read "rec8" (italicised)"
p. 12: "fragmented" AEs/LEs is better than "fractionated".
p. 13: "We analyzed this feature on squashed spermatocytes since the squashing procedure preserves the structure and volume of spermatocyte nuclei." It is true that squashing is better than spreading, but this statement is an extenuation. All that may be said is that squashing preserves the separation of peripheral and internal nuclear domains.
p.14: Correct spelling of the author¥s name is Trelles-Sticken.
p. 14: "However, it has been recently shown that mice lacking the meiosis-specific SMC1fl are defective in telomere capping at prophase I, being this alteration of the bouquet independent of its role in AEs assembly (Adelfalk et al, 2009)." The meaning of this sentence is unclear. My guess is that the functionof SMCb in telomere capping rather than its role in AE assembly may be responsible for the defective bouquet formation in its absence.
"In this aspect, the mild telomeric phenotype we have observed in the absence of RAD21L can be explained by the alteration of those CCs in which this kleisin constitutes a CC together with SMC1fl. However, in the absence of double knock out mice, it can not be excluded that RAD21L directly contributes to this partial alteration in the bouquet or that defects in the pairing and synapsis are the primary cause of this alteration." The meaning of these sentences is unclear. My guess is that they are intended to say that it is unclear if the mild telomere disorganization defect observed is due to the general reduction of functional CCs (caused by the loss of the subset of CCs containing RAD21L) or due to the lack of RAD21L, specifically.
p. 14-15: "At zygotene, strong -H2AX labelling was observed similar to that at the chromatin of both Rad21l-/- and Rad21l+/+ spermatocytes (Figure 7A)." The meaning is unclear should it read that at zygotene, g-H2AX labelling was equally strong in -/- and +/+ spermatocytes or that at zygotene g-H2AX labelling of the sex body was equally strong to autosomal chromatin both in -/- and +/+ spermatocytes?
p. 15: "RAD51 is recruited to these early recombination nodules and participates in the DNA repair mechanism, such as single strand processing, homology recognition, strand invasion, and heteroduplex formation (Ashley, 2008)". Since the activity of RAD51 is well established, you may address this directly by replacing this sentence by "RAD51 is recruited to these early recombination nodules and promotes homologous strand invasion." Also provide a better citation from one of the numerous reviews on the molecular activities of RAD51.
p. 16, line 8: Start a new paragraph with: "We further investigated whether CO and chiasmata..."
p. 16: Add a reference for the effect of okadaic acid.
p. 16, line 22: I have the feeling that "appropriately" would fit better than "accordingly".
p. 16: Wording! "The absence of more than 40 centromeric signals, a maximum of 80 separated chromatids could be obtained, in the RAD21-deficient OA-treated metaphase I-like spermatocytes, is also indicative of the persistence of centromeric cohesion." It should presumably mean: "The observation of 40 rather than 80 centromere signals associated with separated chromatids is indicative of the persistence of centromeric cohesion."
p. 17: "These results, together with the preferential location of RAD21L at the sex AEs at expense of REC8 (Figure 5) and the observed fertility in mutant females, make it tempting to speculate ..." This sentence nicley summarizes the prime message of the paper bu it should come after the results on female rad21l-/- meiosis, because it makes reference to it. Also, replace "sex AEs" by "sex

chromosomal AEs".
p. 17: The authors claim that a cohesion defect was not responsible for the sterility of old rad21L-/- females because "Diakinesis/metaphase I chromosomes from RAD21Ldeficient oocytes were normal and 20 bivalents were always observed." I could not find information on the age of rad21L-/- females in which bivalent formation was checked. Provide the information how many oocytes of how many mice at which ages were assayed.

p. 19: "... the absence of loss of centromeric cohesion ..." Wording! ".... maintenance of centromeric cohesion...."
p.20: Correct spelling of the author¥s name is Tachibana-Konwalski.

Referee #2 (Remarks to the Author):

Review, Herran et al., "The cohesin subunit RAD21L functions in..."

In early 2011, three labs identified a novel kleisin subunit of the cohesin complex, the RAD21L. Cohesin acts in key biological processes in all eukaryotic cells, is essential, but not yet fully understood. The identification of RAD21L as a meiosis-specific subunit was a very important contribution to this field. Now, Herran et al already present the first data on the function of RAD21L in the mouse, i.e. the first data on a RAD21L "KO". This is very remarkable and such in vivo studies are highly important. The data presented by Herran et al are sound and highlight specific and important roles for RAD21L outside of cohesion.

This manuscript thus conveys novel and important results and should be published provided a few improvements were done. These are suggested below.

There are quite a lot of awkward sentences, grammatical problems, and typos in the manuscript, which should be carefully read and edited preferably by a native English speaker. This is more than just "cosmetics", since occasionally the meaning of a sentence is cryptic or even wrong, may cause misunderstandings, and thus this nice paper would suffer from that. This reviewer points out only a few examples of those problems.

In the introduction, references are missing for REC8, SMC1b, STAG3.

The results section (p 6) starts with mentioning "controversial results" in the three 2011 papers on RAD21L. Only a minority of the previously published localization data is controversial. The authors need to precisely describe here, which data are controversial, and thus provide a very specific reason for addressing this problem. As it is now, the reader is left wondering what the controversy is about. It does not help much that at the end of this rather long section, the discrepancies are discussed a bit more, but even then not in sufficient detail.

Next sentence must read ..." further deepen our understanding of the precise localization..." (the localization itself must of course not be "deepened"...)

Page 6: While it is good to use two independent antibodies specific for RAD21L, this does not remove all uncertainties surrounding the localization issue. A tagged version of RAD21L, expressed in a mouse, may further help (but still not be perfect). This would, however, clearly go beyond the scope of this paper, but the authors may want to discuss the "controversy" in very cautious terms. The two antibodies used by Herran et are apparently very specific, since in supplemental figure 1D no signal is seen with one of the antibodies on zygotene-like chromosomes fro RAD21L-deficient mice. It would be reassuring if a Western blot is shown as well loaded with extracts from wt and from ko mice, and if both antibodies were tested. In the previous paper, no data on the antibody specificity were shown.

Even if there would be more antibodies and all be proven specific, the "controversy" would not be entirely solved. Different fixation and staining procedures alone will result in differences. Except for using a tagged transgenic (with the tagged gene expressed from a BAC) and this having its own disadvantages, there are not many more tools available and thus this question will always remain open to some small extend - as is usual in the field.

Apoptosis in the male at stage IV is typical of many meiosis deficiencies and may be triggered through expression of the Zfy1/2 genes (Royo et al., 2010), which is not appropriately silenced anymore, particularly in asynapsis mutants like the RAD21L where the typical sex body does not form, silencing factors do not accumulate there anymore. The authors state that they do not "believe" that Zfy1/2 expression kills spermatocytes in their mutant, but that would need to be shown. Whether one calls the most advanced stage reached in the mutant zygotene-like or early pachytene-like is not really significant. Thus, it would be very good to see Zfy1/2 expression, which can be easily checked by real-time RT-PCR. This would be important for the discussion on page 20/21.

Page 13: The discussion on measurements of stoichiometry in vivo is lengthy; that section can be shortened.

gH2Ax labels DSBs but also unsynapsed regions. Thus, accumulation of gH2AX on autosomes does not necessarily reflect accumulation of DSBs. The authors may like to rephrase the respective sentence (Page 15, top).

MLH1 foci may never form in the mutant, since the cells are dying at the stage of foci formation. How about MSH4?

Typos/errors (examples):
Abstract, 2nd line from bottom: "evidence", not "evidences"

Intro, p.4, 11th line from bottom: the "However" is misplaced here; in fact, it is not necessary and gives the sentence a possibly wrong meaning.

Intro, page 5, line 3 from bottom: "recently", not "recent"

Page 6: "sex chromosome AEs", not "sex chromosomes AEs"

Page 12, line 7 from bottom: remove the "Although"; it conveys a wrong meaning.

Page 13, last sentence first paragraph: awkward sentence, rewrite.

The term "knock-outs" is kind of lab jargon and in most cases not worthy of scientific paper.

Referee #3 (Remarks to the Author):

The manuscript by Herran et al. describes the generation and characterization of a knock out mouse for the RAD21L, and a-kleisin identified earlier this year. This novel cohesin subunit appears to be meiosis specific, and two recent reports have described its localization in spermatocytes. Based on this localization, it has been proposed that cohesin complexes containing RAD21L promote homologue pairing and recombination. The present report provides in vivo validation for this hypothesis and shows that male mice lacking RAD21L-/- are sterile due to defects in synapsis that lead to an arrest in meiotic prophase I followed by apoptosis. Interestingly, and unlike results in mice KO for other meiosis-specific cohesin subunits (Rec8 and Smc1b), sterility is only observed in males, not in females. Nevertheless, fertility of RAD21L-/- females is reduced at much earlier age than in wild types. My overall impression of the quality of the data presented and their relevance is quite positive. My major concern is that the manuscript is very difficult to read in its current format. I therefore have suggestions regarding how to improve its readability as well specific comments on the data that I list below:

Major points:
-Separate Results and Discussion, and make subdivisions within each of these two sections. Each subdivision should address one specific question.

-Results should be presented in a more concise way. Just as an example, I have condensed the description of Figure 1 as follows:

"In order to assess the localization of RAD21L, we carried out a detailed analysis of mouse spermatocytes spreads by immunofluorescence (IF). RAD21L was first detected at leptotene as short threads, formed by small dots, which colocalized with SYCP3 along developing AEs (Figure 1A-D). When pairing between homologues begins in zygotene, RAD21L colocalized with SYCP3 at both the autosomal AEs/LEs, and at the unsynapsed AEs of the sex chromosomes (Figure 1E-H). In early pachytene, both RAD21L and SYCP3 were detected as continuous lines along the autosomal SCs and at the AEs of the sex chromosomes, either synapsed or unsynapsed (Figure 1I and J). By late pachytene there was an increase in RAD21L labeling on the sex chromosomes AEs and on the chromatin of the sex body (Figure 1K and L). This localization is in contrast with the observed weak staining of REC8 at the AEs of the sex chromosome at this stage (see asterisk in panel --- of Figure 5). In early diplotene the intensity of the RAD21L labeling decreased along the desynapsing (it is not clear what we should see this in the image) and still synapsed LEs (Figure 1M-P) to finally disappear by mid diplotene (Figure1Q and R). Concomitantly, RAD21L began to accumulate at centromeres (Figure 1Q-T) while it was progressively lost from the AEs and the chromatin of the sex chromosomes (Figure 1M-T)."

Also in page 7, the descriptions are too long. Why mention: "Interestingly, the RAD21L signal at the centromere of the Y chromosome was larger than at the X chromosome" or later on "Is also interesting to note that RAD21L was not detected at the large SYCP3 agglomerates present in the cytoplasm of metaphase I spermatocytes" if the relevance of these observations is not discussed further? Also, definitions like "the interchromatid domain" could be removed from main text and left in the Figure Legend, or just indicated in the picture.

-In some cases, especially when figures are composed by multiple panels, the reader does not know where to look when going from the main text to the figure. It would help to label individual panels, e.g., in Figure 4 or Figure 5, just as it was done for Figure 1 or 2. Moreover, as a general comment, the authors should try to point the reader better.
For example, in page 7, "However, there was a faint RAD21L signal at the unsynapsed AEs of the sex chromosomes that did not colocalize with the enlargements of SYCP3 (...) (Figure 2A and B, arrowheads)."

In Figure 4A, indicate the reader where to look by specifying the panel and asterisks, arrowheads, arrows, etc in the main text.

-I think the manuscript should be also reorganized regarding the order of the figures. It would make more sense to me if Figures 7 and 8A would follow Figure 4. These figures explore by IF staining which events of a meiotic prophase take place in the mutant and which do not. All along the description of Figure 7 and 8, the text is very repetitive and could be considerably shortened.

-Figure 8B deserves a separate Figure and section, since it leads to an important result: that RAD21L complexes do not play a major role in centromeric cohesion. The description of the experiment in the current manuscript is in page 16 but its discussion appears later, after Figure 9, in page 18-19 (!). Anyway, the authors employ treatment with okadaic acid to bypass the zygotene arrest and thereby reach a pseudometaphase I state. While "treated wild type spermatocytes revealed 20 bivalents, positive for SYCP3 (...) with two pairs of unseparated sister kinetochores (...) Rad21l-/- spermatocytes displayed 40 unattached univalents with a characteristic labeling of SYCP3 only at the centromeric domain (Figure 8B). The absence of more than 40 centromeric signals [indicates] the persistence of centromeric cohesion". My suggestion is to rewrite this as a separate section and to strengthen this interesting result by showing a quantitative analysis (number of centromeric signals/metaphase, n metaphases). It would also be great to stain these pseudometaphases with REC8 and ACA to see that REC8 persists at cetromeres.

-The authors examine in Fig 5 the effect of RDA21L knock down in the localization of other cohesin subunits by IF and observe a clear decrease in STAG3 and, to a lesser extent, in SMC1a. It would be informative to show that this decrease is not stage-specific and occurs throughout prophase I. Moreover, another important point is to repeat the analysis in female germ cells. Maybe the reason for the sexual dimorphism lies, at least in part, in the relative abundance of the distinct cohesin complexes. In this case, Figure 5 could be placed at the end of the Results section.

-Regarding the "telomere attachment" phenotype (Figure 6), ChIP-dot blot analyses with telomeric probes or immunofluorescent staining could be used to demonstrate the presence of RAD21L at telomeres (similar to Adelfalk 2009). Otherwise, I think this section should be removed or at least moved to Supplementary info and its mention in the main text considerably shortened. It could be mentioned in Discussion, as a secondary effect of the "defects in pairing and synapsis" caused by lack of RAD21L.

Minor points:
-I think it is unnecessary and confusing to abbreviate "cohesin complex" by CC. This abbreviation is not used in the field.

-Page 3, Introduction. "In vertebrates, most of the CCs are dissociated from the chromatid arms at prophase through the phosphorylation of the STAG2 subunit by a not very well understood PLK1 mediated mechanism (Waizenegger et al, 2000)" This is not correct. First, both STAG1 and STAG2 are phoshorylated and thereby dissociate in mitotic prophase in vertebrates. Second, the correct references showing this are: (Sumara et al 2002; Losada et al 2002) instead of (Waizenegger et al 2000).

-Page 3, Introduction " This mechanism ultimately enables biorientation of recombined homologues" is redundant with the next sentence [" The persistence of centromeric cohesion ..."]. Delete one or the other.

-Page 4, first line, I guess it should read "In addition to REC8, a meiotic paralogue of RAD21, there are also meiosis-specific paralogues of ..."

-The Introduction should include a more thorough description of other mouse models for meiosis-specific cohesin subunits, i.e., Rec8 and Smc1b.

-At the beginning of Results section, the authors indicate that IF staining of RAD21L was performed with two different antibodies. They should specify in the Figure Legend which of the two antibodies was used in the images shown and, if it is the case, mention that similar or indistinguishable results were obtained with the other one.

-Figure S1, description of the KO. Is an mRNA corresponding to exons 1 to 9 transcribed? Does it result in the translation of C-terminal truncated protein? Clarify the position of the primers used to amplify RAD21L ORF in Fig. S1C and the antigen recognized by the antibody used in S1D.

Referee #4 (Remarks to the Author):

Herran et al report further detailed characterisation at the cytological level of the recently discovered meiotic cohesin component RAD21L. The paper is in two parts. In the first the authors use two antibodies to localise the protein at various stages of meiosis. Two previous papers have described somewhat different results using different antibodies directed against this protein. These discrepancies will only be resolved by exchange of reagents and highlight the problems of immunolocalisation methods but the data presented here support the contention that RAD21 and RAD21L have specific functions.
This is further supported by the generation of a RAD21L null mouse. This is well documented showing synapsis problems in males and to a lesser extend in females. Defects in telomere clustering, lack of DSB repair and absence of Mlh1 foci marking crossover were also present as is the case in many mutations affecting synapsis.
The paper is long and could be shortened substantially which would make the points made easier to extract. Careful editing of the MS would help (there are multiple lapses in English which should be corrected) and some figures are not needed. For example fig 5 in which loading changes in REC8 etc are not detected could be cut to show just the relevant STAG3 SMC1a panels. In this figure I am also not convinced that the wt pachytene should be compared to the null Zygo like null image. Fig 7C could also be omitted since the conclusion is that the nulls are the same as controls. Similarly 8A can be omitted as is is described adequately in a single sentence.
The female null phenotype is well described at the cytological level but there are no details on litter size and total pups born per mother. These might be useful singe if there are synapsis defects in

some oocytes these might not be entirely eliminated during development but might contribute to aneuploid embryos eliminated during uterine development.

1st Revision - authors' response 02 June 2011

Please find enclosed the revised version of the MS entitled "The cohesin subunit RAD21L functions in meiotic synapsis and exhibits sexual dimorphism in fertility".

We have carried out an in depth revision of the MS following the constructive advice of the reviewers. We have changed the order of the presentation of the data and of the figures. We have subdivided the MS in chapters with subheadings. The re-written manuscript has avoided "wording" and has been substantially reduced in those aspects highlighted by the reviewers such as the description of the histology, description of the cytology, discussion of some results and especially in the telomeric analysis. In this regard, we have reduced the length of the telomeric paragraph (reduction of a 50%) and also we have reduced its weight within the MS by transferring the corresponding figure to the supplementary material (now Figure S7).

From an experimental point of view, we have tried to address all the criticisms and concerns raised by the four referees. Thus, we have incorporated novel data (supplementary Figure 2) showing the specificity of the antibodies (by IF and WB) in a heterologous system (293T cells). We also provide novel results using IP procedures to further validate the specificity of the antibodies and to show that in the KO mice RAD21L protein is not detected by western blot (Figure S1E). We incorporate novel data showing that the decrease in the loading of STAG3 and SMC1 occurs similarly throughout prophase I in the spermatogenesis of the KO mice (Figure S5 and S6). In this same regard, we have performed the required experiments to show that STAG3 and SMC1 are not altered in the oocytes of the mutant female mice (Figure S8A). We also provide additional results showing the immunolabeling of the recombinant protein MSH4 in spermatocytes from wild type and KO mice (Figure S4B). Finally, we have also analysed the pseudo-metaphases induced with Okadaic acid with REC8 and ACA and showed that REC8 persists at centromeres (Figure 6B).

We now look forward that the revised MS might be now considered for publication in EMBO J

Referee #1 (Remarks to the Author):

The paper consists of two major parts. One is a relatively straight-forward report of knockout phenotypes which clearly confirm the proposed role of RAD21L (or RAD21L-containing cohesin complexes) in DSB repair, homology searching and synapsis. A remarkable find is that rad21l-/- meiosis is normal in young female mice, but that lack of the protein confers an age-dependent affect on fertility.
The other part is basically a repeat of studies on the cytological localization of RAD21L. This part is justified since two previous papers by two other groups presented partially contradicting results and here, confirmation is provided for the observations in one of the two. However, in one crucial detail, the present manuscript differs from both previous reports. They had described an alternating localization of RAD21L and REC8 along meiotic chromosome axes, whereas here, they are shown to form continuous overlapping stretches. On the basis of the alternating pattern of RAD21L and REC8, one group put forward a model of a locus specific barcode of distribution of the two kleisins that would be shared between homologous chromosomes and provide a means of homology alignment or recognition. Since this is a strong statement, a more rigorous effort should be made here to provide explanations for the controversial observations.
I was wondering if the authors could try to apply antibodies at a lower concentration, since it is possible that immunostaining intensity depends on slightly varying densities of proteins along axial/lateral elements. These differences might be revealed only if antibody binding is not oversaturated.

We appreciate the comment raised by referee#1. Following his/her advice we have performed IF of

spermatocytes at several dilutions. The results we have obtained do not shed more light to this controversy. When the antibodies against RAD21L are diluted above a threshold (1:17) we loose the signal at the AE/LEs of the chromosomes at prophase I (data not shown), leaving the labelling at the centromeres and sex body. However, when we start to detect RAD21L immunolabeling [by increasing the concentration of the antibody (1:15)] the signal appears along the AEs/LEs in a continuous fashion (not shown).
From the beginning of this study (as also commented by Referee#2) we believed that a transgenic mice with a tagged Rad21l expressed from a BAC would be an attractive alternative (more sensitive and specific) to the use of antibodies against RAD21L. Thus, we generated four independent transgenic founders from a RAD21l-EGFP recombineered BAC. However, we have not detected EGFP expression in the transgenic mice (neither by IF nor by IP+WB) despite of positive transgene expression by RT-PCR and northern blot analysis. Strikingly, we observed in the transgenic mice a reduction of the transcription of the endogenous RAD21L gene similar to the low level of transcription for the RAD21L-EGFP transgene. Thus, we have not been able to develop the suitable tools to dissect this aspect of the loading of the kleisins along the AE/LEs of mouse spermatocytes. We have nonetheless incorporated in the revised MS a short technical mention that could account for the observed differences among the studies, leaving always this subject open to further analysis.

While the data presented and the conclusions drawn here are valid and scientifically sound, the manuscript is poorly written. The writing style is overly wordy and convoluted, thus obfuscating rather than clarifying the work. The manuscript would greatly benefit from extensive editing by a native English speaker to present the work clearly and directly.
Also, readability would be improved by a more structured presentation of results and discussion. Subtitles should be used in the corresponding Results & Discussion section. Such chapters could follow the current organization of the section and include: Immunolocalization of the RAD21L protein. Gene disruption of rad21l. SC morphology and synapsis in mutant spermatocytes. Cohesion complexes in mutant spermatocytes. Telomere behaviour in mutant spermatocytes. Defective DSB processing in the mutant. Meiosis in mutant females.

We appreciate his/her advice regarding the organization of the MS. Accordingly, we have ordered the presentation of the MS in chapters to facilitate the reading of the MS. The order of the presentation has also been changed. In addition, we have re-written the MS trying to avoid "wording". The MS has also been reviewed by two native English speakers. We believe that in the present form, the MS is more structured and easy to read.

Finally, in many parts, the manuscript is too copious, e.g., with the description of the testicular histology of rad21L-/- mutant mice, when it is anyway made clear later that the observed anomalies are due to a failure in pairing, SC formation and DSB repair (which are known to lead to apoptosis and azoospermia). Parts of the manuscript read like a review article as much of the discussion barely relates to the findings reported here.

We have reduced the description of the histology of the mutant mice and have also shortened the description of the cytology.

Specific comments:

p. 4, line 20, the yeast mutant should read "rec8" (italicised)"
This has been modified accordingly in the revised version.

p. 12: "fragmented" AEs/LEs is better than "fractionated".
It has been modified accordingly in the revised version.

p. 13: "We analyzed this feature on squashed spermatocytes since the squashing procedure preserves the structure and volume of spermatocyte nuclei." It is true that squashing is better than spreading, but this statement is an extenuation. All that may be said is that squashing preserves the separation of peripheral and internal nuclear domains.

We appreciate the more direct sentence suggested by the referee. It has been modified in the revised version.

p.14: Correct spelling of the author´s name is Trelles-Sticken.
This reference has been eliminated

p. 14: "However, it has been recently shown that mice lacking the meiosis-specific SMC1ß are defective in telomere capping at prophase I, being this alteration of the bouquet independent of its role in AEs assembly (Adelfalk et al, 2009)." The meaning of this sentence is unclear. My guess is that the function of SMCb in telomere capping rather than its role in AE assembly may be responsible for the defective bouquet formation in its absence.
"In this aspect, the mild telomeric phenotype we have observed in the absence of RAD21L can be explained by the alteration of those CCs in which this kleisin constitutes a CC together with SMC1ß. However, in the absence of double knock out mice, it can not be excluded that RAD21L directly contributes to this partial alteration in the bouquet or that defects in the pairing and synapsis are the primary cause of this alteration." The meaning of these sentences is unclear. My guess is that they are intended to say that it is unclear if the mild telomere disorganization defect observed is due to the general reduction of functional CCs (caused by the loss of the subset of CCs containing RAD21L) or due to the lack of RAD21L, specifically.

We apologize for the wrong and several times "baroque" way of writing. We appreciate the efforts made by referee#1 in understanding and in converting the paragraph in an understandable one. Consequently, we have modified the paragraph following his/her advice.

p. 14-15: "At zygotene, strong g-H2AX labelling was observed similar to that at the chromatin of both Rad21l-/- and Rad21l+/+ spermatocytes (Figure 7A)." The meaning is unclear should it read that at zygotene, g-H2AX labelling was equally strong in -/- and +/+ spermatocytes or that at zygotene g-H2AX labelling of the sex body was equally strong to autosomal chromatin both in -/- and +/+ spermatocytes?

We now have incorporated the first interpretation in the text.

p. 15: "RAD51 is recruited to these early recombination nodules and participates in the DNA repair mechanism, such as single strand processing, homology recognition, strand invasion, and heteroduplex formation (Ashley, 2008)". Since the activity of RAD51 is well established, you may address this directly by replacing this sentence by "RAD51 is recruited to these early recombination nodules and promotes homologous strand invasion." Also provide a better citation from one of the numerous reviews on the molecular activities of RAD51.

We appreciate the criticism raised by the referee. We have introduced the correction which alleviates the paragraph. A better citation has been included (Mimitou et al, 2009).

p. 16, line 8: Start a new paragraph with: "We further investigated whether CO and chiasmata..."

We appreciate the comment raised by the referee. We have started a new chapter with its own subheading entitled "Okadaic acid-induced metaphase I-like spermatocytes"

p. 16: Add a reference for the effect of okadaic acid.
The original reference of the use of Okadaic acid has been incorporated (Whiltshire et al, 1995)

p. 16, line 22: I have the feeling that "appropriately" would fit better than "accordingly".
We agree that the term appropriately is more adequate. It has been corrected in the revised version.

p. 16: Wording! "The absence of more than 40 centromeric signals, a maximum of 80 separated chromatids could be obtained, in the RAD21-deficient OA-treated metaphase I-like spermatocytes, is also indicative of the persistence of centromeric cohesion." It should presumably mean: "The observation of 40 rather than 80 centromere signals associated with separated chromatids is indicative of the persistence of centromeric cohesion."

Thanks for the suggestion. It has been corrected in the revised version.

p. 17: "These results, together with the preferential location of RAD21L at the sex AEs at expense of REC8 (Figure 5) and the observed fertility in mutant females, make it tempting to speculate ..." This sentence nicley summarizes the prime message of the paper bu it should come after the results on female rad21l-/- meiosis, because it makes reference to it. Also, replace "sex AEs" by "sex chromosomal AEs".

We agree with the reviewer that the conclusion should not be applied at the end of male mutants, since it makes reference to female fertility. However, we have also simulated to relocate it to the end of the paragraph Meiosis in mutant female mice, and the result is also quite discordant since the conclusion is now too separated from the analysis of male spermatogenesis. Thus, we have left the conclusion in its original position but we have eliminated the reference to female fertility.

p*. 17: The authors claim that a cohesion defect was not responsible for the sterility of old rad21L-/- females because "Diakinesis/metaphase I chromosomes from RAD21Ldeficient oocytes were normal and 20 bivalents were always observed." I could not find information on the age of rad21L-/- females in which bivalent formation was checked. Provide the information how many oocytes of how many mice at which ages were assayed.*

The information about the number and age of the females has been added to the text "To analyze crossovers at diakinesis, we did chromosome preparations of oocytes (n 15 per female) from 3 females of 18 weeks of age from each genotype following the method described previously (Kan et al, 2008)"

p. 19: "... the absence of loss of centromeric cohesion ..." Wording! ".... maintenance of centromeric cohesion...."
Thanks for the suggestion. We have modified the text.

p.20: Correct spelling of the author´s name is Tachibana-Konwalski.
It has been corrected in the revised version.

Referee #2 (Remarks to the Author):

In early 2011, three labs identified a novel kleisin subunit of the cohesin complex, the RAD21L. Cohesin acts in key biological processes in all eukaryotic cells, is essential, but not yet fully understood. The identification of RAD21L as a meiosis-specific subunit was a very important contribution to this field. Now, Herran et al already present the first data on the function of RAD21L in the mouse, i.e. the first data on a RAD21L "KO". This is very remarkable and such in vivo studies are highly important. The data presented by Herran et al are sound and highlight specific and important roles for RAD21L outside of cohesion.

This manuscript thus conveys novel and important results and should be published provided a few improvements were done. These are suggested below.

There are quite a lot of awkward sentences, grammatical problems, and typos in the manuscript, which should be carefully read and edited preferably by a native English speaker. This is more than just "cosmetics", since occasionally the meaning of a sentence is cryptic or even wrong, may cause misunderstandings, and thus this nice paper would suffer from that. This reviewer points out only a few examples of those problems.

The manuscript has been re-written and revised by two independent native English speakers.

In the introduction, references are missing for REC8, SMC1b, STAG3.
These references have now been incorporated in the introduction.

The results section (p 6) starts with mentioning "controversial results" in the three 2011 papers on RAD21L. Only a minority of the previously published localization data is controversial. The authors need to precisely describe here, which data are controversial, and thus provide a very specific reason for addressing this problem. As it is now, the reader is left wondering what the controversy is about. It does not help much that at the end of this rather long section, the discrepancies are discussed a bit more, but even then not in sufficient detail.

We have included at the beginning of the section the most substantial discrepancy between the published works and eliminated the term controversial from the text. In addition, to shorten this section (from 1122 to 825 words) we have reduced the description of the results and discussed the results more shortly.

Next sentence must read ..." further deepen our understanding of the precise localization..." (the localization itself must of course not be "deepened"...)

This sentence have been eliminated to shorten the MS

Page 6: While it is good to use two independent antibodies specific for RAD21L, this does not remove all uncertainties surrounding the localization issue. A tagged version of RAD21L, expressed in a mouse, may further help (but still not be perfect). This would, however, clearly go beyond the scope of this paper, but the authors may want to discuss the "controversy" in very cautious terms. The two antibodies used by Herran et are apparently very specific, since in supplemental figure 1D no signal is seen with one of the antibodies on zygotene-like chromosomes fro RAD21L-deficient mice. It would be reassuring if a Western blot is shown as well loaded with extracts from wt and from ko mice, and if both antibodies were tested. In the previous paper, no data on the antibody specificity were shown.
Even if there would be more antibodies and all be proven specific, the "controversy" would not be entirely solved. Different fixation and staining procedures alone will result in differences. Except for using a tagged transgenic (with the tagged gene expressed from a BAC) and this having its own disadvantages, there are not many more tools available and thus this question will always remain open to some small extend - as is usual in the field.

-We appreciate the comment and suggestion and agree with Referee#2 that a tagged version of RAD21L might shed some light. In collaboration with Dr. Ina Poser and Frank Buchholz, we have developed a modified BAC of RAD21L with a fused EGFP at the C terminus of the protein. Transgenic mice (4 founders) have been generated but, despite the transgene was transcribed, we could not detect EGFP expression. In the absence of these data, and following his/her advice, we have dealt this controversy in more cautious terms. Following his/her recommendation we have included, as suppl. Data (Figure S2), some experiments showing by both IF and western blot that the antibodies we have developed and used in the present study do not cross-react with RAD21 and vice versa. For that purpose, we have transfected the expression plasmids encoding Flag-RAD21 and Flag-RAD21L in 293 and showed the specificity of the polyclonal antibodies by IF and WB. In addition, and following referee#2¥s advice, we have also included immunoprecipitation experiments of wild type and RAD21L-deficient mice showing that RAD21L is not detected in extracts from Rad21l-/- (Figure S1E).

Apoptosis in the male at stage IV is typical of many meiosis deficiencies and may be triggered through expression of the Zfy1/2 genes (Royo et al., 2010), which is not appropriately silenced anymore, particularly in asynapsis mutants like the RAD21L where the typical sex body does not form, silencing factors do not accumulate there anymore. The authors state that they do not "believe" that Zfy1/2 expression kills spermatocytes in their mutant, but that would need to be shown. Whether one calls the most advanced stage reached in the mutant zygotene-like or early pachytene-like is not really significant. Thus, it would be very good to see Zfy1/2 expression, which can be easily checked by real-time RT-PCR. This would be important for the discussion on page 20/21.

We appreciate the criticism raised by the reviewer on the apoptosis at stage IV triggered through the expression of the Zfy1/2 genes. It demonstrates that we were not able to discuss this aspect

appropriately in the text. In the original MS we intended to communicate that we believed that Zfy1/2 expression could be one of the effectors that were causing the generalized apoptosis. Our primary argument was that even in those mutants arrested well before MSCI apoptosis occurs physiologically (mid pachytene), the expression of these pro-apoptotic genes (ZFY1/2) during an indefinite arrest (i.e. zygotene arrest) could be killing the spermatocytes. Thus, the sustained expression of these pro-apoptotic genes at previous stages and not only at pachytene (as a consequence of a defect in the inactivation of these Y-linked genes by the MSCI mechanism) could similarly lead to apoptosis. We have modified the text accordingly and hope that in the present form our argument would be transmitted properly.

Page 13: The discussion on measurements of stoichiometry in vivo is lengthy; that section can be shortened.

We have addressed this point and have drastically reduced the discussion of the stoichiometry section.

gH2Ax labels DSBs but also unsynapsed regions. Thus, accumulation of gH2AX on autosomes does not necessarily reflect accumulation of DSBs. The authors may like to rephrase the respective sentence (Page 15, top).

We do appreciate the comment raised by the reviewer and agree with the consideration that gH2Ax labelling on autosomes does not necessarily reflect accumulation of DSBs. Although, it has been argued that the "weak" gH2AX labelling at zygotene vs the strong labelling at pachytene is due to the presence of unrepaired DSBs and to the existence of asynapsed regions, respectively (Holloway et al, JCB 2010), this assumption is difficult to measure. Thus, we preferred to consider the appreciation made by the reviewer.

MLH1 foci may never form in the mutant, since the cells are dying at the stage of foci formation. How about MSH4?

We had not looked at MSH4, but agree with the referee#2 that it could be of interest to look for the presence of this protein. Thus, we have undertaken IF localization of MSH4 in Rad21l+/+ and Rad21l-/- spermatocyte spreads. As stated in the MS with a new short paragraph and new panel of the Figure S4B, we detected a slight decrease in the signal of MSH4 along the AE/LEs of RAD21L null spermatocytes. The new results have been included in the main text (Defective DSB processing in the mutant spermatocytes) and also as a new supplementary figure (S4).

Typos/errors (examples):
Abstract, 2nd line from bottom: "evidence", not "evidences"

Intro, p.4, 11th line from bottom: the "However" is misplaced here; in fact, it is not necessary and gives the sentence a possibly wrong meaning.

Intro, page 5, line 3 from bottom: "recently", not "recent"

Page 6: "sex chromosome AEs", not "sex chromosomes AEs"

Page 12, line 7 from bottom: remove the "Although"; it conveys a wrong meaning.

Page 13, last sentence first paragraph: awkward sentence, rewrite.

The term "knock-outs" is kind of lab jargon and in most cases not worthy of scientific paper.

We appreciate the indication of the mistakes and their correction. The text has been modified accordingly.

Referee #3 (Remarks to the Author):

The manuscript by Herran et al. describes the generation and characterization of a knock out mouse for the RAD21L, and a-kleisin identified earlier this year. This novel cohesin subunit appears to be meiosis specific, and two recent reports have described its localization in spermatocytes. Based on this localization, it has been proposed that cohesin complexes containing RAD21L promote homologue pairing and recombination. The present report provides in vivo validation for this hypothesis and shows that male mice lacking RAD21L-/- are sterile due to defects in synapsis that lead to an arrest in meiotic prophase I followed by apoptosis. Interestingly, and unlike results in mice KO for other meiosis-specific cohesin subunits (Rec8 and Smc1b), sterility is only observed in males, not in females. Nevertheless, fertility of RAD21L-/- females is reduced at much earlier age than in wild types. My overall impression of the quality of the data presented and their relevance is quite
positive. My major concern is that the manuscript is very difficult to read in its current format. I therefore have suggestions regarding how to improve its readability as well specific comments on the data that I list below:

Major points:
-Separate Results and Discussion, and make subdivisions within each of these two sections. Each subdivision should address one specific question.

We appreciate the comment and suggestion made by the reviewer. We have included most if not all the comments raised by the reviewers in the new MS in order to present the results in a more concise way. We have ordered the presentation of the MS in chapters with subheading and we have also changed the order of two of these chapters in relation with the previous MS to facilitate the reading. Following these corrections, we believe that now the text has considerably improved and can be presented in the format of results and discussion within a single section.

-Results should be presented in a more concise way. Just as an example, I have condensed the description of Figure 1 as follows:

"In order to assess the localization of RAD21L, we carried out a detailed analysis of mouse spermatocytes spreads by immunofluorescence (IF). RAD21L was first detected at leptotene as short threads, formed by small dots, which colocalized with SYCP3 along developing AEs (Figure 1A-D). When pairing between homologues begins in zygotene, RAD21L colocalized with SYCP3 at both the autosomal AEs/LEs, and at the unsynapsed AEs of the sex chromosomes (Figure 1E-H). In early pachytene, both RAD21L and SYCP3 were detected as continuous lines along the autosomal SCs and at the AEs of the sex chromosomes, either synapsed or unsynapsed (Figure 1I and J). By late pachytene there was an increase in RAD21L labeling on the sex chromosomes AEs and on the chromatin of the sex body (Figure 1K and L). This localization is in contrast with the observed weak staining of REC8 at the AEs of the sex chromosome at this stage (see asterisk in panel --- of Figure 5).

In early diplotene the intensity of the RAD21L labeling decreased along the desynapsing (it is not clear what we should see this in the image) and still synapsed LEs (Figure 1M-P) to finally disappear by mid diplotene (Figure1Q and R). Concomitantly, RAD21L began to accumulate at centromeres (Figure 1Q-T) while it was progressively lost from the AEs and the chromatin of the sex chromosomes (Figure 1M-T)."

Also in page 7, the descriptions are too long. Why mention: "Interestingly, the RAD21L signal at the centromere of the Y chromosome was larger than at the X chromosome" or later on "Is also interesting to note that RAD21L was not detected at the large SYCP3 agglomerates present in the cytoplasm of metaphase I spermatocytes" if the relevance of these observations is not discussed further? Also, definitions like "the interchromatid domain" could be removed from main text and left in the Figure Legend, or just indicated in the picture.

We have followed these recommendations and used this rewritten text. Thus, we have reduced/eliminated from the text long descriptions in order to make the MS more fluid. This section

in the former version included 1122 words and now includes 825 words.

-In some cases, especially when figures are composed by multiple panels, the reader does not know where to look when going from the main text to the figure. It would help to label individual panels, e.g., in Figure 4 or Figure 5, just as it was done for Figure 1 or 2. Moreover, as a general comment, the authors should try to point the reader better.
For example, in page 7, "However, there was a faint RAD21L signal at the unsynapsed AEs of the sex chromosomes that did not colocalize with the enlargements of SYCP3 (...) (Figure 2A and B, arrowheads)."
In Figure 4A, indicate the reader where to look by specifying the panel and asterisks, arrowheads, arrows, etc in the main text.

Following the criticism raised by the reviewer, we have incorporated throughout the text where the reader has to look by specifying the panel/asterisks of the figure. To do that, we have also subdivided Figures 6 and 7 in multi-pannels.

-I think the manuscript should be also reorganized regarding the order of the figures. It would make more sense to me if Figures 7 and 8A would follow Figure 4. These figures explore by IF staining which events of a meiotic prophase take place in the mutant and which do not. All along the description of Figure 7 and 8, the text is very repetitive and could be considerably shortened.

We have shortened the description of the former Figure 7 and 8 in order to avoid repetitions. Following his/her criticism, we have changed the description order in the main text corresponding to the subheadings "Defective DSB processing in the mutant spermatocytes" and "Okadaic acid-induced metaphase I-like spermatocytes", thus the former Figure 7 and 8 are now converted to figure 5 and 6.

-Figure 8B deserves a separate Figure and section, since it leads to an important result: that RAD21L complexes do not play a major role in centromeric cohesion. The description of the experiment in the current manuscript is in page 16 but its discussion appears later, after Figure 9, in page 18-19 (!). Anyway, the authors employ treatment with okadaic acid to bypass the zygotene arrest and thereby reach a pseudometaphase I state. While "treated wild type spermatocytes revealed 20 bivalents, positive for SYCP3 (...) with two pairs of unseparated sister kinetochores (...) Rad21l-/- spermatocytes displayed 40 unattached univalents with a characteristic labeling of SYCP3 only at the centromeric domain (Figure 8B). The absence of more than 40 centromeric signals [indicates] the persistence of centromeric cohesion".
My suggestion is to rewrite this as a separate section and to strengthen this interesting result by showing a quantitative analysis (number of centromeric signals/metaphase, n metaphases). It would also be great to stain these pseudometaphases with REC8 and ACA to see that REC8 persists at cetromeres.

We appreciate referee#3's comment on this section. We have accordingly separated this part and created a new section with its own discussion extracted and modified from the former page 18-19. We have included the number of cells and individuals analysed. In addition, we have also performed Rec8/ACA staining of OA-Induced metaphase I-like cells. The new results (incorporated in Figure 6B) show that REC8 labels the centromeres in both wild type and RAD21L-deficient metaphase 1-like, sustaining the notion that centromeric cohesion is not altered

-The authors examine in Fig 5 the effect of RAD21L knock down in the localization of other cohesin subunits by IF and observe a clear decrease in STAG3 and, to a lesser extent, in SMC1a. It would be informative to show that this decrease is not stage-specific and occurs throughout prophase I. Moreover, another important point is to repeat the analysis in female germ cells. Maybe the reason for the sexual dimorphism lies, at least in part, in the relative abundance of the distinct cohesin complexes. In this case, Figure 5 could be placed at the end of the Results section.

Following this comment of referee#3, we have included novel data indicating that the decrease in STAG3 labelling is not restricted to a single stage. This novel figure is included in supplementary material as Figure S5.

We have also carried out the analysis of STAG3 and SMC1 in the female prophase I, showing that in oogenesis both STAG3 and SMC1 are apparently not decreased in the absence of RAD21L. This new results have been incorporated in a new Figure S8A. We have also included this result in the "Meiosis mutant female" paragraph to highlight the parallelism between a proper STAG3/ SMC1 loading and fertility in females.

-Regarding the "telomere attachment" phenotype (Figure 6), ChIP-dot blot analyses with telomeric probes or immunofluorescent staining could be used to demonstrate the presence of RAD21L at telomeres (similar to Adelfalk 2009). Otherwise, I think this section should be removed or at least moved to Supplementary info and its mention in the main text considerably shortened. It could be mentioned in Discussion, as a secondary effect of the "defects in pairing and synapsis" caused by lack of RAD21L.

We appreciate the suggestion of this reviewer in relation to the use of ChIP dot blot analysis, which is of interest to further investigate in detail the telomeric phenotype. Following also the opportunity provided by the editor in this respect, we have considered that lightening this part of the MS could be more realistic given that this is only a subtle aspect of the meiotic phenotype observed in the mice.
We have reduced the weight of the telomeric analysis in the MS by reducing its content (reduction of 50%) and also by eliminating part of the discussion in relation with REC8 (including the elimination of three references).

Minor points:
-I think it is unnecessary and confusing to abbreviate "cohesin complex" by CC. This abbreviation is not used in the field.

In order to avoid confusion and following the referee¥s indication we have eliminated the abbreviation CC

-Page 3, Introduction. "In vertebrates, most of the CCs are dissociated from the chromatid arms at prophase through the phosphorylation of the STAG2 subunit by a not very well understood PLK1 mediated mechanism (Waizenegger et al, 2000)" This is not correct. First, both STAG1 and STAG2 are phoshorylated and thereby dissociate in mitotic prophase in vertebrates. Second, the correct references showing this are: (Sumara et al 2002; Losada et al 2002) instead of (Waizenegger et al 2000).

We appreciate the comment raised by the reviewer which indicates that we have not properly written the intended concept and that we have cited a wrong reference. The term "by a not well understood PLK1-mediated mechanisms" was referring to the mechanism by which the cohesin complex is dissociated from the chromatids in prophase as a consequence of the phosphorylation and not to the phosphorylation of STAG1 and STAG2 by PLK1. We have modified the text trying to avoid this problem. Our wrong reference has also been changed for the correct ones, as suggested by the referee

-Page 3, Introduction " This mechanism ultimately enables biorientation of recombined homologues" is redundant with the next sentence [" The persistence of centromeric cohesion ..."]. Delete one or the other.

It has been modified accordingly in the revised version by deleting the second of the two redundant sentences.

-Page 4, first line, I guess it should read "In addition to REC8, a meiotic paralogue of RAD21, there are also meiosis-specific paralogues of ..."
It has been modified in the text.

-The Introduction should include a more thorough description of other mouse models for meiosis-specific cohesin subunits, i.e., Rec8 and Smc1b.
We have included a short paragraph with a description of the two other mutants for the meiotic

cohesin subunits (Rec8 and SMC1B). The description of the mutants was very summarized due to space restrictions and because of the presence of a more thorough discussion of these mutants in page 18.

-At the beginning of Results section, the authors indicate that IF staining of RAD21L was performed with two different antibodies. They should specify in the Figure Legend which of the two antibodies was used in the images shown and, if it is the case, mention that similar or indistinguishable results were obtained with the other one.

We used both antibodies to validate that the results obtained could be reproduced with either of them. In addition, we have included in the Material and Methods section a sentence about this point: "Both polyclonal antibodies against RAD21L (GutiÈrrez-Caballero et al, 2011) were used to validate the results of the IF and westen blot data presented throughout this work". In this regard, we have included additional experimental data to support the specificity of the antibodies in new supplemental figures (Figure S1E and Figure S2A and S2B).

-Figure S1, description of the KO. Is an mRNA corresponding to exons 1 to 9 transcribed? Does it result in the translation of C-terminal truncated protein? Clarify the position of the primers used to amplify RAD21L ORF in Fig. S1C and the antigen recognized by the antibody used in S1D.

Following the referee¥s advice, we have included the sequence of the primers and their position which correspond with the ATG and STOP codons of RAD21L (Figure S1A). We have also indicated in the supplementary material a description of the location of the antigens used to generate both antibodies and the exons by which are encoded. We can not exclude that exons 1-9 could be transcribed and translated. However, and in the event they were expressed, the targeted mutation generated would cause the loss of the C-terminus end of the protein (exons 10-13). In any case, this would lead to a non-functional truncated protein lacking the essential C-terminus domain that closes the ring by interacting with the ATPase head of SMC1 (Haering et al, 2004). In addition, binding of the C-terminal domain of REC8 and RAD21 to SMC1 is essential for the binding of the N-terminal domain to SMC3 and for the formation of a functional cohesin complex (Arumugan et al, 2006; Haering et al, 2004). This aspect is included in the supporting material in the paragraph corresponding to "Targeting the murine Rad21l locus and creation of mutant mice". It is also remarkable to note that Rad21l+/- spermatocytes/oocytes/mice have been analysed throughout the whole experimental procedure being indistinguishable from the Rad21l+/+spermatocytes/oocytes/mice, thus eliminating a gain of function allele.

Referee #4 (Remarks to the Author):

Herran et al report further detailed characterisation at the cytological level of the recently discovered meiotic cohesin component RAD21L. The paper is in two parts. In the first the authors use two antibodies to localise the protein at various stages of meiosis. Two previous papers have described somewhat different results using different antibodies directed against this protein. These discrepancies will only be resolved by exchange of reagents and highlight the problems of immunolocalisation methods but the data presented here support the contention that RAD21 and RAD21L have specific functions.
This is further supported by the generation of a RAD21L null mouse. This is well documented showing synapsis problems in males and to a lesser extend in females. Defects in telomere clustering, lack of DSB repair and absence of Mlh1 foci marking crossover were also present as is the case in many mutations affecting synapsis.
The paper is long and could be shortened substantially which would make the points made easier to extract. Careful editing of the MS would help (there are multiple lapses in English which should be corrected) and some figures are not needed. For example fig 5 in which loading changes in REC8 etc are not detected could be cut to show just the relevant STAG3 SMC1a panels. In this figure I am also not convinced that the wt pachytene should be compared to the null Zygo like null image. Fig 7C could also be omitted since the conclusion is that the nulls are the same as controls. Similarly 8A can be omitted as is is described adequately in a single sentence.

The whole MS have been rewritten and shortened following the referee¥s advice.
We have eliminated the former Figure 7C from the MS and sent it to the supplementary material since there are no differences in RPA in null versus controls. We have also eliminated from the main MS the figure corresponding to the telomeric analysis. Following this same criteria, we have not eliminated former Figure 8A (present Figure 6A), since the absence of MLH1 in the RAD21L-deficient spermatocytes is different from the wild type controls. Similarly, we have maintained the panels of present Figure 7 in order to allow the direct comparison of loading changes in STAG3 and SMC1 to the remaining subunits which do not show differences in their loading (Rec8, Rad21 etc).

The female null phenotype is well described at the cytological level but there are no details on litter size and total pups born per mother. These might be useful singe if there are synapsis defects in some oocytes these might not be entirely eliminated during development but might contribute to aneuploid embryos eliminated during uterine development.

We have included the information we have available in relation with the average number of offspring per litter during their period of fertility (1.5-6 months; with no differences between genotypes) and during the period of subfertility up to infertility (6-10 months; 5.2 in Rad21l-/- vs 8.9 in Rad21l+/+). The reduction in the size of the litter can not however discern between exhaustion of the oocyte pool or intrauterine foetal loss.

Acceptance letter 16 June 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal. All the referee still asks for are four minor textual modifications; please briefly make these changes and return only the modified text document via email at your earliest convenience. If you have not already done so, please also send us the necessary copyright forms at this stage in order to avoid delays with the production process.

Once we will have received these things, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

Referee #2

(Remarks to the Author)
Herran et al, revised version of "The cohesin subunit RAD21L..."

The authors have adequately addressed the points raised in the initial review, and the paper should now be published after only very few changes. These are:

Page 15: the authros did not study "loading" of cohesins, but determined the presence of cohesin on chromosomes. In other words, the authors describe the status quo, and not the process of loading. Thus, they should replace the word "loading" by "presence" or "existence" ... of cohesin on chromsomes/SCs etc.

Page 16: I do not think that Adelfalk et al determined a specific "capping" function for cohesin, but

rather described more general telomere protection. Protection may be provided through other means than only capping. Thus, the word"capping" does not seem to be very appropriate.

Page 19: for the explanation of sexual dimorphism, a key topic of this paper, the authors may want to take the recent important paper by the Hunt lab into account (Nagoaka et al., Curr Biol., 2011).

Page 7: "imagecapture" should be "image capture" or "capturing of images", or even better: "image acquisition"

